INFECTION AND IMMUNITY, Apr. 2005, p. 2135–2146 Vol. 73, No. 4
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.4.2135–2146.2005

Regulation of Type III Secretion Hierarchy of Translocators and Effectors in Attaching and Effacing Bacterial Pathogens

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Received 20 September 2004/Returned for modification 9 November 2004/Accepted 3 December 2004

Human enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and the mouse pathogen *Citrobacter rodentium* (CR) belong to the family of attaching and effacing (A/E) bacterial pathogens. They possess the locus of enterocyte effacement (LEE) pathogenicity island, which encodes a type III secretion system. These pathogens secrete a number of proteins into culture media, including type III effector proteins and translocators that are required for the translocation of effectors into host cells. Preliminary evidence indicated that the LEE-encoded SepL and Rorf6/SepD may form a molecular switch that controls the secretion of translocators and effectors in CR. Here, we show that SepL and SepD indeed perform this function in A/E pathogens such as EHEC and EPEC. Their *sepL* and *sepD* mutants do not secrete translocators but exhibit enhanced secretion of effectors. We demonstrate that SepL and SepD interact with each other and that both SepL and SepD are localized to the bacterial membranes. Furthermore, we demonstrate that culture media influence the type III secretion profile of EHEC, EPEC, and CR and that low-calcium concentrations inhibit secretion of translocators but promote the secretion of effectors, similar to effects on type III secretion by mutations in *sepL* and *sepD*. However, the secretion profile of the *sepD* and *sepL* mutants is not affected by these culture conditions. Collectively, our results suggest that SepL and SepD not only are necessary for efficient translocator secretion in A/E pathogens but also control a switch from translocator to effector secretion by sensing certain environmental signals such as low calcium.

One of the emerging themes in bacterial pathogenesis is that diverse gram-negative pathogenic bacteria employ a conserved protein secretion machinery termed the type III secretion system (TTSS) as a major virulence mechanism to cause disease in their hosts (11). These bacteria include some of the most important and devastating human and plant pathogens in the genera *Yersinia*, *Salmonella*, *Escherichia*, *Shigella*, *Pseudomonas*, and *Xanthomonas*. The TTSS is a multiprotein complex evolutionarily related to the flagellar apparatus, and it consists of more than 20 proteins that form a so-called needle complex spanning both the inner and outer membranes of the bacterial envelope. It is postulated that the TTSS apparatus acts as a molecular syringe, injecting effector proteins (effectors) from the bacterial cytosol directly into the host cell cytoplasm, where the effectors act to facilitate bacterial proliferation and disease development (9, 11).

Although the exact numbers can vary, many pathogens secrete a large number of proteins via the TTSS, most of which fall into two categories, effectors and translocators (7, 9, 21). The effectors modulate host cellular functions and signal transduction pathways and subvert host defense mechanisms upon injection into host cells. The translocators are not needed for type III secretion (TTS) but are required for translocating effectors into host cells by assembling a translocation conduit in the host cell membrane. While each pathogen may possess a unique assortment of effectors to suit its own pathogenesis strategy and specific host, translocators are generally conserved among the pathogens. Because translocators are required for the injection of effectors into host cells, it is assumed that the pathogens have evolved mechanisms to ensure that translocators are secreted prior to effectors, so that effectors will be exported directly into the targeted host cells instead of the extracellular milieu. However, the control of this process is poorly understood (9).

TTS is thought to be triggered by direct bacterial contact with the host cell, but the nature of this contact remains controversial (9). Many environmental factors modulate TTS, inducing the secretion of effectors into culture media. For example, low-calcium conditions induce TTS of effector Yops in *Yersinia* spp. (29), and the dye Congo red triggers TTS of Ipa proteins in *Shigella flexneri* (2, 39). However, whether these environmental cues serve as authentic signals during infections is unknown. Understanding the molecular mechanism of how these signals trigger TTS should lead to better appreciation of the control and regulation of TTS in bacterial pathogenesis.

Human diarrheagenic enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), the mouse pathogen *Citrobacter rodentium* (CR), and many EPEC and EHEC isolates from pets and farm animals belong to the family of attaching and effacing (A/E) pathogens. These pathogens cause much morbidity and mortality worldwide and represent significant threats to human and animal health (6, 36).

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A/E pathogens share a pathogenicity island called the locus of enterocyte effacement (LEE) that encodes a TTSS. A needle complex similar to that found for *Salmonella* and *Shigella* spp. has been visualized in EPEC (43, 48). The needle complex of EPEC displays an extracellular filamentous extension made of EspA, one of the secreted proteins (26, 43). The LEE-encoded TTSS is essential for virulence of these pathogens (6, 15, 37). A/E pathogens secrete several LEE-encoded proteins into culture media using the LEE-encoded TTSS, including EspA, EspB, EspD, EspF, EspG, EspH, Tir, and Map (6, 15, 46). These secreted proteins are divided into effectors (EspF, EspG, EspH, Tir, and Map) and translocators (EspA, EspB, and EspD). In addition, A/E pathogens secrete a number of non-LEE-encoded effectors (5, 15, 23, 31, 32, 35, 47). One of them is NleA/EspI, which is targeted to the host cell Golgi and is critical for bacterial virulence (23, 35).

As for many TTSSs in other bacteria, the molecular mechanism for the hierarchy control of translocator and effector secretion in A/E pathogens is poorly understood. It has been reported that calcium modulates TTS in A/E pathogens as well (24, 25). Calcium chelation of growth medium reduces TTS of the translocators and increases the secretion of effector Tir in EPEC and diffusely adhering EPEC strains by an unknown mechanism (24, 25). We have recently shown that LEE-encoded SepL and Rorf6 differentially regulate the secretion of translocators and effectors in CR and may represent a TTS hierarchy switch (15). Rorf6 has been renamed SepD (38); based on our results, we follow this nomenclature in this report. However, there are conflicting reports with regard to the function of SepL and especially SepD in EPEC and EHEC (27, 38). While needed for translocator but not effector secretion in CR (15), SepD has been reported to be an essential element of the TTSS in EPEC, required for both translocator and effector secretion (38). In this report, we demonstrate that both SepL and SepD are essential for the secretion of translocators but not effectors and that the SepL/SepD hierarchy switch from translocator secretion to effector secretion is a conserved feature in all A/E pathogens, including CR, EHEC, and EPEC. In addition, we show that calcium limitation suppresses secretion of translocators but enhances general secretion of effectors in EPEC, EHEC, and CR, suggesting a link between calcium sensing and the SepL/SepD switch.

MATERIALS AND METHODS

CR strains and LEE gene deletion mutants. The CR wild-type (WT) strain and its deletion mutants of single LEE genes, including *espA*, *espD*, *espB*, *sepL*, *sepD*, and *escN*, were described previously (14, 15). To create a triple-deletion mutant of CR *espA*, *espD*, and *espB*, two PCR fragments that covered the upstream region of *espA* and the downstream region of *espB* were amplified with PCR primer pairs (CRespADB-F1 and CRespADB-R1; CRespADB-F2 and CRespADB-R2) (Table 1). The two PCR fragments were ligated into the KpnI/SacI-digested *sacB* gene-based suicide vector pRE118 (18). The resulting plasmid contained 2.5 kb of the upstream region of *espA* and 2.1 kb of the downstream region of *espB*, with deletion of the *espA*, *espD*, and *espB* genes in between. This plasmid was introduced into CR by electroporation, and in-frame triple-deletion mutants of *espA*, *espD*, and *espB* were generated by sucrose selection as described previously (14, 18).

Generation of *sepL* and *sepD* nonpolar deletion mutants in EHEC and EPEC. Both *sepL* and *sepD* genes are located within polycistronic operons, at least in EPEC (34). In CR, EPEC, and EHEC O157:H7, the predicted stop codon of *sepD* overlaps in the nucleotide sequence TAATG with the start codon of the downstream *escC* that encodes an essential component of the TTS apparatus (22), suggesting possible translational coupling. To avoid any polar effects on the expression of genes downstream of *sepL* and *sepD*, we employed the *sacB* gene-based allelic exchange method to generate in-frame *sepL* and *sepD* internal deletion mutants in both EPEC and EHEC. The suicide vector pRE112 (18) was used to generate deletion mutants in the nalidixic acid-resistant (Nal^r) derivative

TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′) (restriction enzyme)[a]
CRespADB-F1	GGTACCGCTTCGAAGAGGTAGAAGATG (KpnI)
CRespADB-R1	GCTAGCCATCGATGTCGAAGTACTCGC (NheI)
CRespADB-F2	GCTAGCCGTATGGGGCAATCGGCTCG (NheI)
CRespADB-R2	GAGCTCTAATCTAAAGCATCAGCAGGG (SacI)
HsepL-F1	GGGGTACCCCGTGCTGAATCGGACATCAG (KpnI)
HsepL-R1	CCGCTAGCAGTGAGCAGAGAGAGAATGCA (NheI)
HsepL-F2	GGGCTAGCAACAGATGCGGGGTTTTGATT (NheI)
HsepL-R2	CCGAGCTCTGGGCGGGAGATGTTGATACC (SacI)
HsepD-F1	CCGGTACCATGCCGCTGGCGACCTCACTC (KpnI)
HsepD-R1	CCGCTAGCCCAGTCACAATCATTCTTTGC (NheI)
HsepD-F2	GGGCTAGCATGATGCCGCCAACACACTTG (NheI)
HsepD-R2	CCGAGCTCTGCAGATTTTATCGGGGTTGC (SacI)
PsepL-F1	GCGCATGCTTCGTCTGGGCGGGAGATGTT (SphI)
PsepL-R1	GCGCTAGCCGTTTCAGAAAGCGATGAGCT (NheI)
PsepL-F2	GCGCTAGCGCATCATTACCTGATGCTTGT (NheI)
PsepL-R2	GCGAGCTCCACTCGCGCAGAATCAGACAT (SacI)
PsepD-F1	CCGCATGCTGCAGATTTTATCGGGGTTGC (SphI)
PsepD-R1	GCGCTAGCATGATGCCGCCAACACACTTG (NheI)
PsepD-F2	CCGCTAGCAAGGGCCGTTAGCCAGTCACA (NheI)
PsepD-R2	CCGAGCTCGCAGTATTACCACTGGCAGCC (SacI)
F1-EscN	CCGGTACCCGCAGCCAGAACATCCGTGAC (KpnI)
R1-EscN	GGGCTAGCACGATGGGGCAAGATCCTGAAG (NheI)
F2-EscN	GGGCTAGCTGCCGGCACAGTGCTATTGAG (NheI)
R2-EscN	CCGAGCTCCACCGCAGGCGTGGTTGTTAC (SacI)
CRsepL-F	GGATCCCATCCTGGTGGTTGAACAATGAG (BamHI)
CRsepL-R	GTCGACCATCGATGTCGAAGTACTCGC (SalI)
CRsepD-F	GGATCCAGTGTCGTTATCAAGTCATCC (BamHI)
CRsepD-R	GTCGACGAGAGGATGGGGCCGCCTGAG (SalI)
ECsepL-F	GGATCCCATCCTGGTGGTTGAACAATGAG (BamHI)
ECsepL-R	GTCGACTTGATGTATCCATGTATATACCTCTT (SalI)
ECsepD-F	GGATCCGTGTTGTTATCAAGTCATCCTCAG (BamHI)
ECsepD-R	GTCGACCCAGAGAGGACGGGGCAGCTTGTG (SalI)
GST-CRsepD-F	GAATTCATGAACAATAATAATGGTAGCGCT (EcoRI)
GST-CRsepD-R	CTCGAGTTACACAATTCGTCCTATATCAGA (XhoI)
GST-CRsepL-F	GAATTCATGGCTAATGGTATTGAATTTAATC (EcoRI)
GST-CRsepL-R	CTCGAGTCACATAACATCCTCCTTATAATCG (XhoI)
EPsepD-Nde	TTCCATATGAACAATAATAATGGCATAGC (NdeI)
EPsepD-Bam	CGGGATCCGGGGGCAGCTTGTGCACTGC (BamHI)
EPsepD-Nco	GCCATGGAGAATAATAATGGCATAGCAAAG (NcoI)
EPsepD-Xho	GCTCGAGCACAATTCGTCCTATATCAG (XhoI)
EPsepL-Nde	TTCCATATGGCTAATGGTATTGAATTTAATC (NdeI)
EPsepL-Bam	CGGGATCCATGCTGTAGTTGATGTATCC (BamHI)
EPespFHA-F	GGATCCTCTGAGAGTTAGCCAAGATTAG (BamHI)
EPespFHA-R	CTCGAGCCCTTTCTTCGATTGCTCATAG (XhoI)
CRespFHA-F	GGATCCTCTGAATAGTAAATACAGAAGTATGG (BamHI)
CRespFHA-R	CTCGAGTCCATTTTTAAATTGCTGATAGGC (XhoI)
CRtir-F	GAGCTCGGAAATGCAATTCCG
CRtir-R	GACGAAACGTTCAACTCCCGG
CRespA-F	ATGGATACATCAACTATGAC
CRespA-R	CCGGTTATTTGCCAATGGG
CRespB-F	ATGAATACTATCGATTATAAC
CRespB-R	TTACCCAGCTAAGCGAGC

[a] Restriction site for the enzyme indicated is underlined.

of EHEC O157:H7 strain 86-24 and the streptomycin-resistant (Sm^r) derivative of EPEC O127:H6 strain E2348/69.

To make an internal deletion mutant of *sepL* in EHEC, PCR was used to generate two fragments with primer pairs HsepL-F1 and HsepL-R1 and HsepL-F2 and HsepL-R2 (Table 1). The amplified products were cloned into pCR2.1-TOPO (Invitrogen) and verified by DNA sequencing. After digestion with KpnI/NheI and NheI/SacI, respectively, the two fragments were gel purified and cloned in a three-way ligation into the suicide vector pRE112 restricted with KpnI/SacI. The resulting plasmid pRE-ΔEHsepL contained 1 to 2 kb of flanking regions on both sides of *sepL* and the *sepL* gene with an internal in-frame deletion from nucleotides 43 to 987 (90% of the coding region). An NheI site was introduced into the deletion site. Plasmid pRE-ΔEHsepL was transformed into *E. coli* SM10λ*pir* by electroporation and introduced into EHEC strain 86-24 Nal^r by conjugation. After sucrose selection, EHEC colonies resistant to sucrose and sensitive to chloramphenicol were screened for deletion of *sepL* by PCR. The EHEC *sepL* mutants were then verified by multiple PCRs. Similarly, a nonpolar deletion mutant of *sepD* was generated in EHEC with primer pairs HsepD-F1 and HsepD-R1 and HsepD-F2 and HsepD-R2 (Table 1). The coding region from nucleotides 43 to 408 (80%) of EHEC *sepD* was deleted and replaced with an NheI restriction site.

The same protocol was used to generate internal deletion mutants of *sepL* and *sepD* in EPEC strain E2348/69 Sm^r, with the PCR fragments cloned into SphI/SacI-digested pRE112. The primer pairs of PsepL-F1 and PsepL-R1 as well as PsepL-F2 and PsepL-R2 (Table 1) were used to make the EPEC *sepL* deletion mutant. The coding region from nucleotides 178 to 957 (74%) of the EPEC *sepL* gene was deleted and replaced by an NheI site. The primer pair PsepD-F1 and PsepD-R1 and PsepD-F2 and PsepD-R2 (Table 1) were used for making the EPEC *sepD* deletion mutant. The coding region from nucleotides 55 to 408 (78%) of the EPEC *sepD* gene was deleted and replaced by an NheI site.

Generation of a nonpolar deletion mutant of *escN* and double mutants of *sepL-escN* and *sepD-escN* in EHEC. To make an internal deletion mutant of *escN* in EHEC strain 86-24 Nal^r, PCR was used to generate two fragments with the primer pairs F1-EscN and R1-EscN and F2-EscN and R2-EscN (Table 1). The amplified products were cloned into pCR2.1-TOPO and verified by DNA sequencing. After digestion with KpnI/NheI and NheI/SacI, respectively, the two fragments were gel purified and cloned in a three-way ligation into pRE112 restricted with KpnI/SacI. The resulting plasmid pRE-ΔEH*escN* contained 1 to 2 kb of flanking regions on both sides of *escN* and the EHEC *escN* gene with an internal in-frame deletion from nucleotides 76 to 1206 (85% of the coding region). An NheI site was introduced into the deletion site. Plasmid pRE-ΔEH*escN* was used to generate a Δ*escN* mutant of EHEC by the same protocol described above, and it was also introduced into EHEC Δ*sepL* and Δ*sepD* single mutants to create Δ*sepL*Δ*escN* and Δ*sepL*Δ*escN* double mutants. All the mutants were verified by multiple PCRs.

Construction of plasmids for complementation of CR, EPEC, and EHEC *sepL* and *sepD* mutants. The coding regions and the immediate upstream regions of CR, EPEC, and EHEC were amplified by PCR with the following pairs of primers (Table 1): CRsepL-F and CRsepL-R for CR *sepL*, CRsepD-F and CRsepD-R for CR *sepD*, ECsepL-F and ECsepL-R for EPEC and EHEC *sepL*, and ECsepD-F and ECsepD-R for EPEC and EHEC *sepD*. All the PCR products were first cloned into pCR2.1-TOPO, verified by DNA sequencing, and then subcloned into pACYC184 (New England Biolabs) as a BamHI/SalI fragment.

Bacterial fractionation and protein localization. CR SepL and SepD were tagged with a double hemagglutinin (2HA) epitope at the carboxyl termini with the vector pTOPO-2HA (15). The 2HA-tagged SepL and SepD were expressed in both CR and EPEC WT strains. Bacterial cell fractionation was carried out as described for EPEC (22). Mouse monoclonal antibodies (mMAbs) against the HA tag (Covance, Princeton, N.J.) were used for Western blot detection of SepL-2HA and SepD-2HA. The following proteins were used as markers to confirm the integrity of the bacterial fractions, as described previously (22): maltose-binding protein (MBP) for the periplasm, DnaK for the cytosol, Etk for the inner membrane, and intimin for the outer membrane.

Protein-protein interactions. Three methods (bacterial two-hybrid, glutathione *S*-transferase [GST] pulldown, and coexpression and copurification) were used to assay protein-protein interactions between SepL and SepD.

(i) Bacterial two-hybrid. Plasmids from the Stratagene Bacteriomatch Two-Hybrid system vector kit were used for the assay (17). Briefly, PCR was used to incorporate either a 5′-flanking EcoRI and 3′-flanking BamHI site (pBT-bait vector; chloramphenicol resistant[Cm^r]) or a 5′-flanking BamHI and 3′-flanking XhoI site (pTRG-target vector; tetracycline resistant [Tet^r]) to the EPEC open reading frames (ORFs) of *sepD* and *sepL*. Plasmids were cotransformed into XL1 Blue MRF′ (kanamycin resistant [Kan^r]) and selected on the following antibiotic concentrations (in parentheses, in micrograms per milliliter): kanamycin (50), chloramphenicol (5), tetracycline (10), and carbenicillin (0 to 300). Positive interaction turns on the *bla* gene, and the transformant is resistant to carbenicillin (Cb^r). Transformation controls were performed on plates with 50 μg of kanamycin/ml, 50 μg of chloramphenicol/ml, and 10 μg of tetracycline/ml. Interaction controls were also performed by cotransforming with empty pBT and empty pTRG vectors.

(ii) GST pulldown. CR ORFs of *sepD* and *sepL* were PCR amplified with the following primer pairs (Table 1): GST-CRsepD-F and GST-CRsepD-R for *sepD* and GST-CRsepL-F and GST-CRsepL-R for *sepL*. The PCR fragments were cloned into pCR2.1-TOPO and sequenced before being subcloned into EcoRI/XhoI-digested pET-28a(+) (Novagen) and pGEX-6p-1 (Pharmacia) to make N-terminal His and GST fusions to CR SepD and SepL, respectively. Protein binding between GST-SepD and His-SepL, as well as between GST-SepL and His-SepD, was assayed by standard GST pulldown protocols (Pharmacia Biotech). Proteins were detected by Western blotting with antibodies against GST or His.

(iii) Coexpression and copurification. EPEC ORFs of *sepD* and *sepL* were amplified by PCR, cloned into pCR2.1-TOPO, verified by DNA sequencing, and then subcloned into pET-28a(+) to generate His-tagged versions of the proteins and into pET-21a (Novagen) to create untagged versions. EPEC *sepD* was cloned into NdeI/BamHI-digested pET21a with primers EPsepD-Nde and EPsepD-Bam for PCR. EPEC *sepD* was also cloned into NcoI/XhoI-restricted pET-28a with PCR primers EPsepD-Nco and EPsepD-Xho (Table 1). Similarly, EPEC *sepL* was cloned into NdeI/BamHI-digested pET-21a and pET-28a with the same pair of primers EPsepL-Nde and EPsepL-Bam (Table 1). *E. coli* BL21(DE3) cells were transformed with two plasmids expressing an untagged protein (either SepL or SepD from pET-21a) and a His-tagged protein (either SepD or SepL from pET-28a). Overnight cultures in Lurai-Bertani (LB) containing kanamycin and ampicillin were diluted 1:100 into 50 ml of LB with both antibiotics and grown at 37°C with shaking until the optical density value at 600 nm (OD_{600}) reached approximately 0.8. Cultures were then moved to room temperature and induced with 0.5 mM isopropylthio-β-galactoside (IPTG). After overnight shaking incubation, the cells were pelleted and sonicated in 0.6 ml of 50 mM Tris (pH 8) and 150 mM NaCl. The soluble fraction after spinning at 14,000 rpm was added to 50 μl of Ni^{2+} agarose beads (QIAGEN) and mixed together for 1 h at 4°C. Beads were pelleted at 5,000 rpm, the unbound fraction was removed, and four washing steps (each step, 1.4 ml and 10 min of mixing at 4°C) were performed with 50 mM Tris (pH 8) and 150 mM NaCl. A further series of five washing steps was done with 50 mM Tris (pH 8), 150 mM NaCl, and 20 mM imidazole used in each step. A sample was removed from the first imidazole wash. Finally the proteins were eluted from the beads with 50 mM Tris (pH 8), 150 mM NaCl, and 300 mM imidazol. All samples were analyzed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE).

TTS assays. Secreted proteins of CR, EPEC, and EHEC were analyzed as described previously (14, 30). Briefly, bacterial strains were grown overnight in LB plus appropriate antibiotics and then subcultured at a dilution of 1:50 into prewarmed, Dulbecco's modified Eagle's medium (DMEM) or modified M9 minimal medium (30). We typically grew 4 ml of cultures in 10-ml test tubes for CR and EPEC strains that secreted easily detectable amounts of proteins and 25 ml of cultures in 250-ml flasks for EHEC strains that secreted much smaller amounts of proteins. The bacteria were induced for TTS in a CO_2 (5%) tissue culture incubator without shaking for 6 h. Secreted proteins were precipitated with 10% trichloroacetic acid from the culture supernatant and analyzed by SDS-PAGE and Western blotting by ECL Western blotting (Amersham).

Analysis of TTS of EspF in CR and EPEC *sepL* and *sepD* mutants. The coding regions and their immediate upstream regions of CR and EPEC *espF* genes were amplified by PCR, cloned into pCR2.1-TOPO, verified by DNA sequencing, and then subcloned as a BamHI/XhoI fragment into BglII/XhoI-digested vector pCRespG-2HA/BglII (15) to engineer a 2HA epitope tag at the carboxyl termini. The expression of the 2HA-tagged *espF* genes was under the control of the CR *espG* gene promoter in the 2HA tagging vector (15). The primers used for PCR were EPespFHA-F and EPespFHA-R for EPEC *espF* and CRespFHA-F and CRespFHA-R for CR *espF* (Table 1). The 2HA-tagged CR and EPEC *espF* constructs (pCRespF-2HA and pEPespF-2HA, respectively) were introduced into and expressed in CR and EPEC WT strains, as well as their *escN*, *sepD*, and *sepL* mutants, and TTS of CR and EPEC EspF-2HA was analyzed as described above. mMAbs against the HA tag (Covance) were used for Western blot detection of EspF-2HA.

Reverse transcription-PCR and ELISA analysis of expression of *tir*, *espA*, and *espB* genes. To monitor the expression of *tir*, *espA*, and *espB* genes, various CR, EHEC, and EPEC strains were treated similarly to the growth conditions as described above for TTS assays. After induction in DMEM or reconstructed calcium-free DMEM for 6 h in a 5% CO_2 tissue culture incubator, the bacterial

cultures were centrifuged, and the culture supernatant was used for quantitative analysis of secreted EspA, EspB, and Tir by enzyme-linked immunosorbent assay (ELISA), while the bacterial pellet was used for isolating total RNA with the RNeasy Mini RNA purification kit (QIAGEN). The RNA samples were treated with DNase I (1 U of DNase I per 1 μg of RNA) to remove any contaminating DNA. Equal amounts of the total RNA (1 μg) for each strain were then used for reverse transcription (RT) with Superscript reverse transcriptase and random primers from Invitrogen according to the supplier's instructions. The resulting cDNA was used for PCR analysis of *tir*, *espA*, and *espB* expression with gene-specific primers for CR, EPEC, and EHEC. We used the following primers for CR *tir*, *espA*, and *espB* genes (Table 1): for *tir*, CRtir-F and CRtir-R (a 745-bp PCR product); for *espA*, CRespA-F and CRespA-R (a 580-bp PCR product); and for *espB*, CRespB-F and CRespB-R (a 960-bp PCR product). The primers for the respective genes of EPEC and EHEC were designed similarly to the CR genes with regard to the locations of the primers, but their sequences were specific for EPEC or EHEC. The following PCR cycles were used: a 10-min denaturing step at 95°C and 25 to 35 cycles, each cycle consisting of 45 s at 94°C, 45 s at 55°C, and 90 s at 72°C. The PCR products were analyzed by 1% agarose gel electrophoresis. For ELISA analysis of secreted Tir and EspB in bacterial culture supernatant, rat polyclonal antibodies against CR Tir were used to detect CR Tir, mMAbs against EPEC Tir were used to detect EPEC and EHEC Tir, and mMAbs against EPEC EspB were used to detect EspB from CR, EPEC, and EHEC strains.

Effect of calcium concentrations on TTS. Three methods were used to determine the effect of calcium concentrations on TTS profiles of CR, EPEC, and EHEC: (i) addition of calcium chelators into DMEM, (ii) reconstituted DMEM, and (iii) addition of calcium into M9 medium.

(i) Addition of calcium chelators into DMEM. EGTA and 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid (BAPTA) from Sigma were added at concentrations from 1 to 4 mM into DMEM to chelate calcium. The following equation was used to determine free calcium concentration in bacterial growth media as a function of chelator concentration: $0 = [Ca^{2+}]_{free} + ([chelator] - [Ca^{2+}]_{total} + K_d) \times \{[Ca^{2+}]_{free} - ([Ca^{2+}]_{total} + K_d)\}$.

This relationship was solved by the quadratic equation, where: $x = [Ca^{2+}]_{free}$, $A = 1$, $B = ([chelator] - [Ca^{2+}]_{total} + K_d)$, and $C = -([Ca^{2+}]_{total} + K_d)$. The K_d of ($[Ca^{2+}]$ − EGTA) is 56 nM, and the K_d of ($[Ca^{2+}]$ − BAPTA) is 160 nM. The initial (prechelator) $[Ca^{2+}]_{total}$ in DMEM (Invitrogen) was assumed to be 1.8 mM.

(ii) Reconstituted DMEM. Various concentrations (0.5 to 2 mM) of $CaCl_2$ were added to calcium-free DMEM (Invitrogen) to induce protein secretion by CR, EPEC, and EHEC as described above in "TTS assays."

(iii) Addition of calcium into M9 medium. There is no calcium in modified M9 medium (30). Various concentrations (0.5 to 2 mM) of $CaCl_2$ were added to M9. However, precipitates formed when >1 mM of $CaCl_2$ was added, due to the phosphate buffer system used for M9 (data not shown). To avoid this problem, 0.1 M of HEPES (pH 7.4) was used to replace the phosphates in M9.

RESULTS

SepL and SepD control the TTS hierarchy of translocators and effectors in CR. During our systematic mutagenesis studies of all 41 LEE genes in CR, we found that mutations in either *sepL* or *sepD* abolished TTS of translocators EspA, EspB, and EspD but significantly enhanced TTS of effectors such as Tir and NleA (15) (Fig. 1A). A double mutant of *sepL* and *sepD* exhibited the same secretion phenotype as their single mutants. However, the translocators were expressed in the *sepL* and *sepD* mutants. Not surprisingly, the *sepL* and *sepD* mutants produced and secreted, but failed to translocate, Tir into host cells as assayed by immunofluorescence microscopy (data not shown), consistent with the essential role of the translocators in translocating effectors into host cells (6, 20). Our results suggest that SepL and SepD not only are essential for translocator secretion, but also control the TTS hierarchy of translocators and effectors. As expected, both *sepL* and *sepD* were critical for CR virulence in mice (15).

SepL and SepD perform the same function in human A/E pathogens EPEC and EHEC. To extend our observations from CR to prototypical human EPEC and EHEC strains and to determine whether SepL and SepD perform the same roles in different A/E pathogens, we constructed nonpolar *sepL* and *sepD* deletion mutants in both EPEC O127:H6 strain E2348/69 and EHEC O157:H7 strain 86-24 and examined TTS of the mutants. When grown in DMEM, both EHEC and EPEC WT strains secreted the three major proteins EspA, EspB, and EspD (the translocators) (Fig. 1B and C). Under these same growth conditions, EHEC secreted the same profile, but a smaller amount, of these proteins (approximately fivefold less) than EPEC and CR (data not shown). In contrast, the *sepL* and *sepD* mutants of both EHEC and EPEC showed no secretion of the translocators but significantly increased secretion of effectors Tir and NleA that were easily detectable by SDS-PAGE and Coomassie staining (Fig. 1B and C). More-sensitive methods, such as Western blotting, were usually needed to detect these effectors secreted by EHEC and EPEC WT strains. Indeed, WT EPEC secreted at least 500-fold-more EspA and EspB proteins than its *sepL* and *sepD* mutants by ELISA. The amount of EspA and EspB secreted by the EPEC *sepL* and *sepD* mutants was barely detectable even by Western blotting and was only slightly more than that secreted by the negative control type III mutant Δ*escN*, as estimated by ELISA (data not shown). On the other hand, the EPEC *sepL* and *sepD* mutants secreted at least 300-fold-more Tir than WT EPEC (Fig. 1C; ELISA data not shown). These TTS phenotypes of the EPEC and EHEC *sepL* and *sepD* mutants were identical to those of the corresponding CR mutants (Fig. 1A) (15), indicating that SepL and SepD perform the same function in these three A/E pathogens.

We also analyzed the secretion of another LEE-encoded effector, EspF, in CR and EPEC *sepL* and *sepD* mutants, since the role of SepL and especially SepD in EspF secretion was controversial (see the introduction). By doing proteomic analysis of secreted proteins, we previously observed that the *sepL* and *sepD* mutants of both CR and EHEC were able to secrete EspF, Map, Tir, EspG, and EspH along with other non-LEE-encoded effectors such as NleA (15; S. Gruenheid, W. Deng, and B. B. Finlay, unpublished results). However, a recent report showed that an EPEC *sepD* mutant behaved similarly to a TTS mutant, unable to secrete either EspF or the translocator EspB (38). To further support our proteomic data, we generated constructs containing CR or EPEC *espF* with a C-terminal 2HA tag under the control of CR *espG* gene promoter and expressed CR EspF-2HA and EPEC EspF-2HA in CR and EPEC WT strains as well as their Δ*escN*, Δ*sepL*, and Δ*sepD* mutants. As shown in Fig. 1D, both CR EspF-2HA and EPEC EspF-2HA, as well as their Δ*sepL* and Δ*sepD* mutants, were type III secreted by WT but not the type III mutant Δ*escN*. In addition, enhanced secretion of CR EspF-2HA was observed in CR Δ*sepL* and Δ*sepD* mutants (Fig. 1D), although increased secretion of EspF-2HA was not as obvious in EPEC, likely due to the use of the heterologous CR *espG* promoter in driving the EPEC EspF-2HA expression. These results indicated that SepD, similar to SepL, is required for TTS of translocators but not effectors (including EspF) in A/E pathogens.

Complementation of CR, EHEC, and EPEC *sepL* and *sepD* deletion mutants. The *sepL* and *sepD* genes are part of the polycistronic operons *LEE4* and *LEE2*, respectively, as shown in EPEC (34). We attempted to complement their deletion

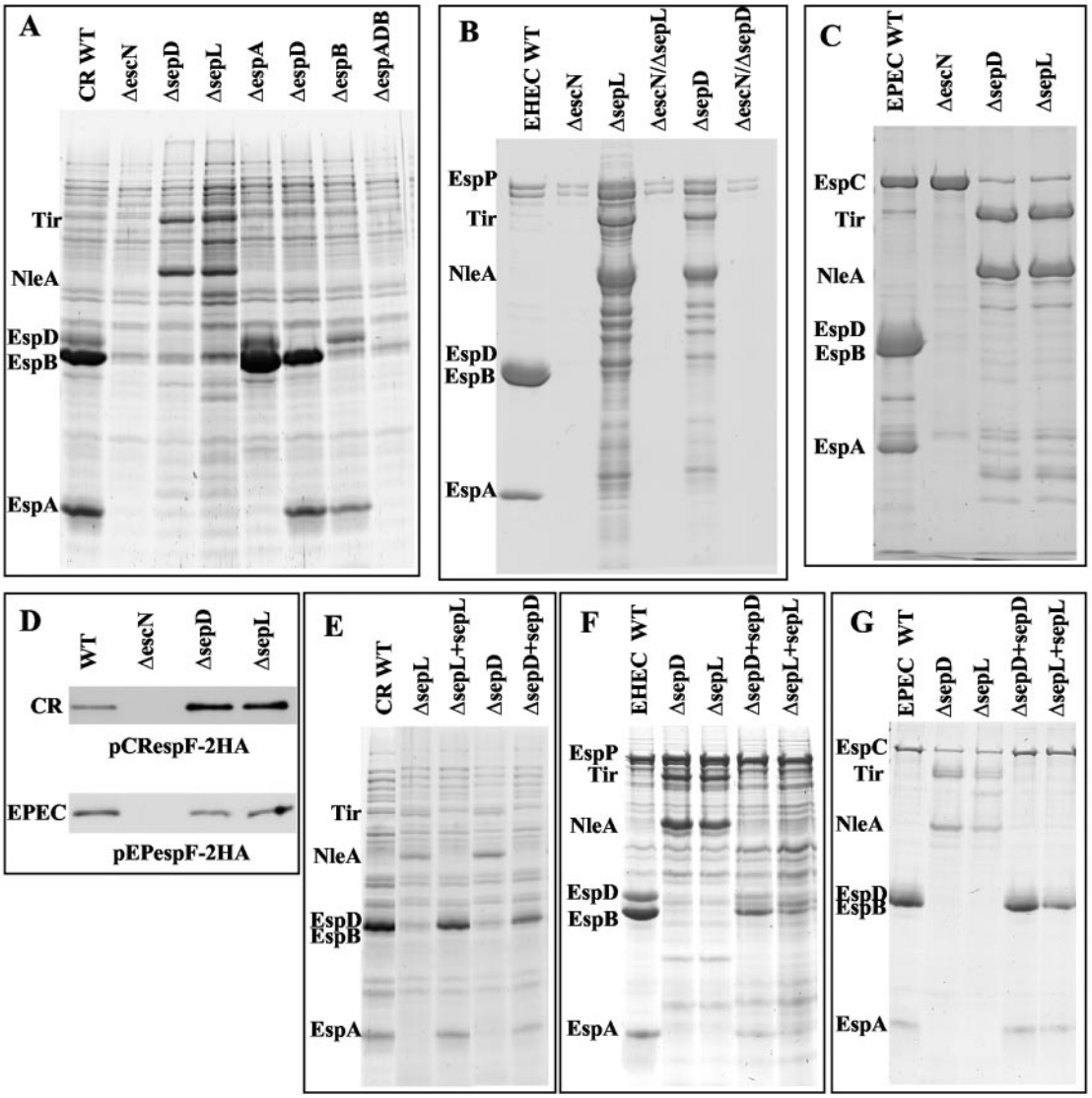

FIG. 1. SepL and SepD perform the same function in CR, EHEC, and EPEC; mutations in their *sepL* and *sepD* genes can be complemented and nonpolar. TTS protein profiles of WT strains and various deletion mutants are shown for CR (A), EHEC O157:H7 strain 86-24 (B), and EPEC O127:H6 strain E2348/69 (C). Also shown are secreted protein profiles of CR (E), EHEC (F), and EPEC (G) Δ*sepD*, Δ*sepL*, Δ*sepD* complemented with *sepD* (Δ*sepD*+*sepD*) and Δ*sepL* complemented with *sepL* (Δ*sepL*+*sepL*) strains. Secreted proteins were concentrated from supernatants of bacterial cultures grown in DMEM and analyzed by SDS–10 to 15% PAGE and Coomassie blue G250 staining. Proteins secreted by equal numbers of bacteria, as estimated by OD_{600} values, were loaded in each lane for each gel. The locations of translocators EspA, EspB, and EspD and effectors Tir and NleA are indicated at the left of each gel. Also indicated are the locations of EPEC EspC and EHEC EspP, which were not secreted via the LEE-encoded TTSS. (D) Both CR and EPEC *sepD* and *sepL* mutants were able to type III secrete EspF-2HA. Plasmids pCRespF-2HA and pEPespF-2HA expressing 2HA-tagged CR EspF-2HA and EPEC EspF-2HA were introduced into WT, Δ*escN*, Δ*sepD*, and Δ*sepL* strains of CR and EPEC. These strains were grown in regular DMEM. Shown are Western blots of concentrated secreted proteins from equal numbers of bacteria, as estimated by OD_{600} values, separated by SDS–10% PAGE, blotted onto nitrocellulose membranes, and probed with anti-HA mMAbs.

mutants with the respective WT genes to rule out the possibility that the translocator secretion defect in the mutants was due to polar effects of the mutations on downstream genes involved in TTS. As shown in Fig. 1E to G, the secretion of translocators EspA, EspD, and EspB was restored, while the secretion of Tir and NleA was suppressed in the complemented strains of all three pathogens when grown in DMEM, resembling the secretion profiles of the respective WT strains. Thus, the mutations in the *sepL* and *sepD* mutants of CR, EHEC, and EPEC were all nonpolar. These data indicated that the hypersecretion of effectors and abolished secretion of translocators in the mutants result directly from the loss of SepL and SepD, suggesting that SepL and SepD control a switching mechanism from secretion of translocators to secretion of effectors.

A triple mutant of *espA*, *espD*, and *espB* in CR does not exhibit enhanced secretion of effectors. It was possible that SepL and SepD are required only for the secretion of translocators and that the significantly enhanced secretion of effectors seen in the CR, EPEC, and EHEC *sepL* and *sepD* mutants was

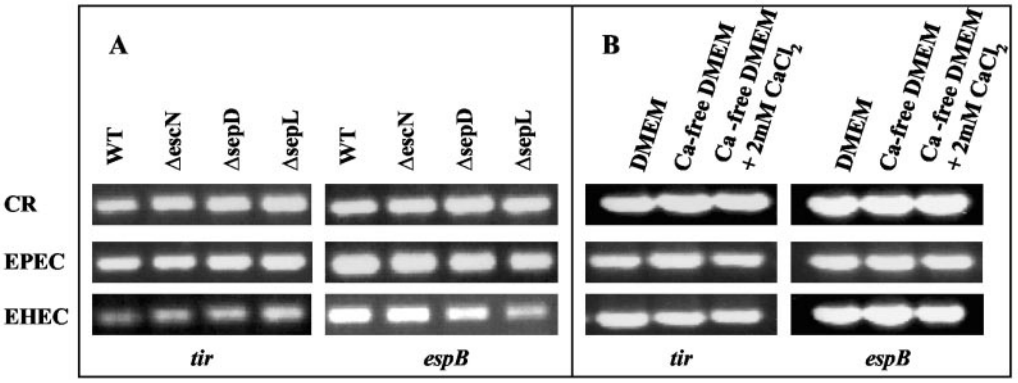

FIG. 2. Transcription of effector Tir and translocator EspB in EHEC, EPEC, and CR is not affected by mutations in *escN*, *sepD*, and *sepL* or by calcium concentrations in the culture medium. (A) CR, EPEC, and EHEC WT and *escN*, *sepD* and *sepL* mutants were grown in LB and subcultured in DMEM to induce LEE gene expression. Total RNA was isolated and treated with DNase I to remove contaminating DNA. Equal amounts of RNA were used to perform RT-PCR, and PCR products were analyzed by agarose gel electrophoresis. There was no PCR product when the RNA samples were directly used for PCR without RT first, suggesting no contamination of chromosomal DNA in the RNA samples (data not shown). (B) WT CR, EPEC, and EHEC strains were grown in LB and subcultured into regular DMEM, reconstituted calcium-free DMEM, and calcium-free DMEM supplemented with 2 mM $CaCl_2$ to induce LEE gene expression. Transcription of *tir* and *espB* was then analyzed by RT-PCR, as described above for panel A.

the result of reduced competition for the secretion channel, due to the abolished translocator secretion. To test this hypothesis, we created a triple *espA*, *espD*, and *espB* deletion mutant (Δ*espADB*) in CR and analyzed its TTS of effectors in DMEM. As shown in Fig. 1A, enhanced secretion of effectors Tir and NleA was observed only in the *sepL* and *sepD* mutants but not in the single mutants of *espA*, *espB*, and *espD* or their triple mutant Δ*espADB*. This demonstrated that the translocators EspA, EspB, and EspD are not required for SepL and SepD to control effector secretion, suggesting that SepL and SepD play an active role in repressing the secretion of effectors and facilitating the secretion of translocators, thereby determining the substrate hierarchy.

Both SepL and SepD modulate secretion, but not transcription, of translocators and effectors. Abolished secretion of EspA, EspB, and EspD and increased secretion of Tir and NleA by the *sepL* and *sepD* mutants could be due to differential expression or differential secretion of the proteins. To determine where SepL and SepD exert their control over the TTS of translocators and effectors, we undertook RT-PCR analysis of *tir*, *espA*, and *espB* transcription in WT, *escN*, *sepL* and *sepD* mutants of CR, EPEC, and EHEC. No significant difference in transcription of *tir*, *espB*, or *espA* was found between the WT strains and their various mutants, including the type III mutant Δ*escN* (Fig. 2A; data not shown). This suggests that SepL and SepD control TTS of translocators and effectors at a posttranscriptional level. However, although they were expressed within the bacteria, EspB and Tir did not accumulate to greater amounts in the *escN* mutant when not secreted; similarly, EspB did not accumulate in the *sepL* and *sepD* mutants (15; data not shown). This suggests a possible posttranscriptional control at the level of translational regulation or an equilibrium between protein synthesis and degradation when the proteins are not secreted.

Both SepL and SepD are localized to the bacterial membranes. Our previous results have shown that neither SepL nor SepD was type III secreted in CR (15), suggesting that they function inside the bacteria. We therefore analyzed the cellular localization of both SepL and SepD in CR and EPEC. For this purpose, we tagged CR SepL and SepD at the carboxyl terminus with a 2HA tag (15) and expressed the tagged proteins in both CR and EPEC. Both constructs could complement their respective mutants (data not shown), suggesting that the 2HA tag did not affect the function and cellular localization of SepL

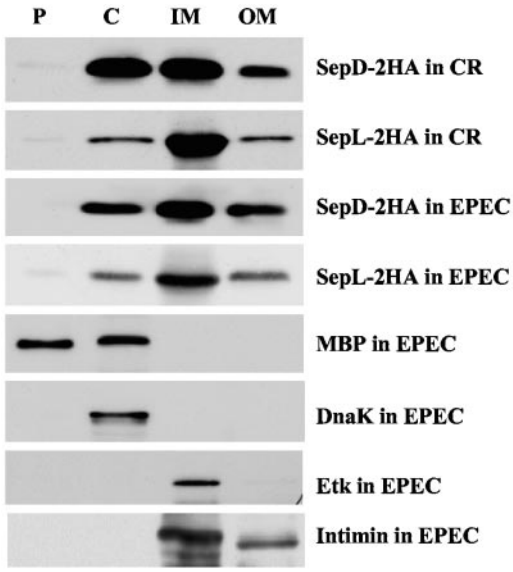

FIG. 3. SepL and SepD are localized mostly to the inner membranes in EPEC and CR. WT EPEC and CR expressing 2HA-tagged CR SepD and SepL were grown in DMEM and fractionated into periplasmic (P), cytoplasmic (C), inner membrane (IM), and outer membrane (OM) fractions. Samples (10 μg per fraction) were analyzed by SDS–10% PAGE and Western blotting and probed with anti-HA mMAbs. The same blots for EPEC fractions were also probed with anti-MBP, anti-DnaK, anti-Etk, and anti-intimin antisera to monitor for contamination of bacterial fractions.

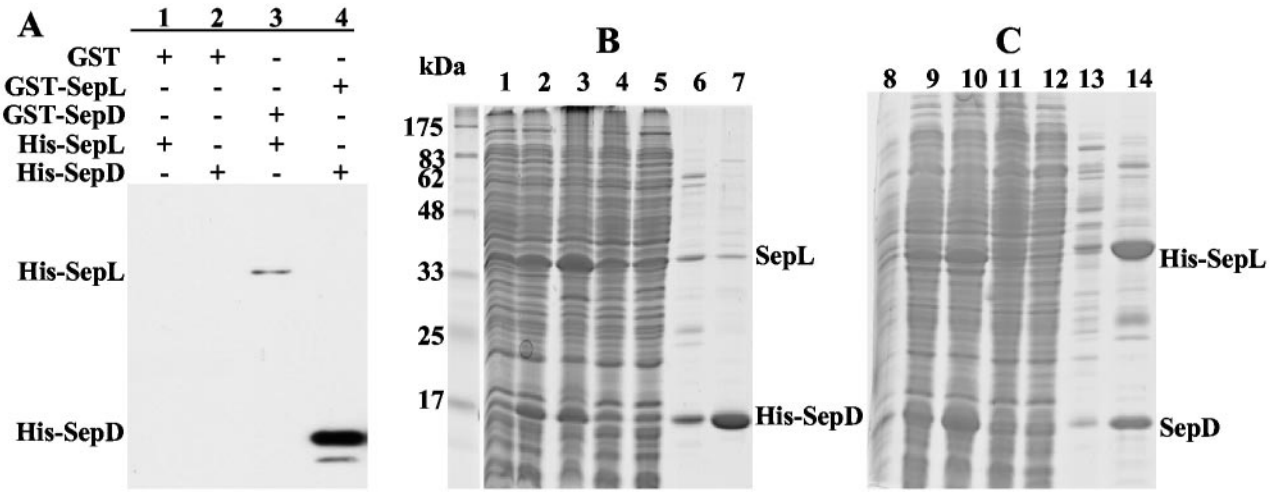

FIG. 4. SepL and SepD interact with each other. (A) GST pull-down assay. Whole-cell lysates from *E. coli* strains expressing GST alone (lanes 1 and 2) or N-terminal GST fusions to CR SepD (lane 3) or SepL (lane 4) were incubated with total-cell lysates of *E. coli* strains expressing N-terminally His-tagged CR SepD (lanes 2 and 4) and SepL (lanes 1 and 3), respectively. After incubation, the lysates were mixed with glutathione-agarose beads (G-beads). The G-beads were then pelleted and extensively washed, and proteins bound to the G-beads were subjected to SDS-PAGE analysis and Western blotting and probed with anti-His antiserum. (B and C) EPEC SepL and SepD bound to each other during coexpression and copurification. The proteins were resolved by SDS–15% PAGE and stained with Coomassie blue G250. (B) Lanes 1 to 7, pET28a EPEC *sepD* C-terminal His tag plus pET21a *sepL*. Lanes 1, Preinduction; 2, overnight induction; 3, insoluble fraction; 4, soluble fraction; 5, unbound fraction; 6, first imidazole wash; and 7, eluted fraction. (C) Lanes 8 to 14: pET28a *sepL* N-terminal His tag plus pET21a *sepD*. Lanes 8, preinduction; 9, overnight induction; 10, insoluble fraction; 11, soluble fraction; 12, unbound fraction; 13, first imidazole wash; and 14, eluted fraction.

and SepD. Bacterial fractionation and Western blot analysis using antibodies against the HA tag showed that both SepL and SepD were present in the cytoplasmic, inner, and outer membrane fractions, with the majority localized to the inner membrane (Fig. 3). The integrity of the fractions was verified by localizing proteins known to be present in a particular bacterial fraction: MBP for the periplasm, DnaK for the cytosol, Etk for the inner membrane, and intimin for the inner and outer membranes (Fig. 3). Our results indicated that SepL and SepD have similar localization profiles in both CR and EPEC.

SepL and SepD interact with each other. Both *sepL* and *sepD* mutants in CR, EPEC, and EHEC display the same secretion phenotype (this study), and their double mutant has the same secretion phenotype as the single mutants in CR (Fig. 1) (15). This suggests that SepL and SepD act in concert and may interact directly. Indeed, three independent methods (bacterial two-hybrid assay, GST pulldown, and coexpression and copurification) showed that SepL and SepD interact with each other (Fig. 4). In the bacterial two-hybrid assay (data not shown), the cotransformants containing the *sepL* and *sepD* constructs conferred resistance to carbenicillin at concentrations of up to 350 μg/ml, and either SepL or SepD could be expressed in either the bait or target vectors. Some carbenicillin resistance was conferred with SepD expressed from both bait and target vectors, suggesting that SepD functions in a multimeric state. To verify the SepL-SepD interaction, we performed GST pulldown assays by generating N-terminal fusions of CR SepL and SepD to GST and expressed the fusion proteins in *E. coli*. As shown in Fig. 4A, GST-SepL and GST-SepD pulled down N-terminally His-tagged CR SepD and SepL, respectively, from crude bacterial lysates, whereas GST alone did not. The SepL-SepD interaction was further confirmed by a coexpression and copurification method. N-terminally His-tagged EPEC SepL was coexpressed with untagged EPEC SepD in the same *E. coli* host. Purification of His-SepL with nickel agarose beads also purified untagged SepD. When the reverse experiment coexpressing C-terminally His-tagged EPEC SepD and untagged SepL was performed, untagged SepL was copurified with SepD-His as well during nickel affinity purification (Fig. 4B and C). The nickel agarose beads failed to bind either untagged SepD or untagged SepL when they were expressed alone in the same host (data not shown). Collectively, these results showed convincingly that SepL and SepD interact with each other and form a protein complex, consistent with our data showing that they had similar cellular localization within the bacteria (Fig. 3).

Culture media influence TTS profiles of A/E pathogens, partially mimicking the effects of *sepL* and *sepD* mutations. We observed that EHEC O157:H7 strains exhibit drastically different secretion profiles in different culture media such as DMEM and modified M9. As shown in Fig. 5, EHEC predominantly secreted translocators EspA, EspD, and EspB in DMEM. However, the secretion of the translocators was much reduced in M9, while greatly enhanced secretion of effectors (Tir and NleA) was observed. EPEC (Fig. 5) and CR (data not shown) WT strains showed similar changes in profiles of TSS proteins when grown in DMEM and M9. On the other hand, the *sepL* and *sepD* mutants of EHEC, EPEC, and CR exhibited the same secreted protein profile in both DMEM and M9 (Fig. 5; data not shown for CR). Interestingly, the secretion profile of WT EHEC, EPEC, and CR in M9 showed significant similarities to that of their *sepL* and *sepD* mutants in both DMEM and M9. The only detectable difference was that the WT

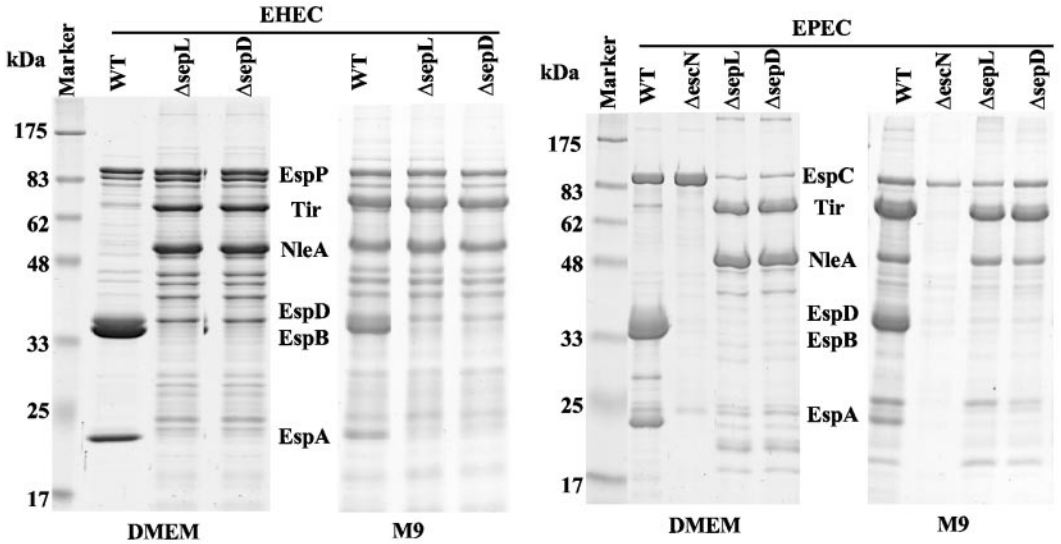

FIG. 5. Culture media differentially modulate TTS of translocators (EspA, EspB, and EspD) and effectors (Tir and NleA) by WT EHEC and EPEC strains but not their *sepL* and *sepD* mutants. WT EHEC and EPEC strains as well as their *escN*, *sepD*, and *sepL* mutants were grown in LB and then subcultured into DMEM or M9 media to induce TTS. Secreted proteins were concentrated from the bacterial culture supernatant and analyzed by SDS-PAGE and Coomassie blue G250 staining. Proteins secreted by an equal number of bacteria, as estimated by OD_{600} values, were loaded in each lane in the same gel. The locations of EspA, EspB, EspD, Tir, and NleA are indicated. EspC (EPEC) and EspP (EHEC) are also indicated, but their secretion was not mediated by the LEE-encoded TTSS.

strains secreted some EspA, EspB, and EspD in M9, while the *sepL* and *sepD* mutants did not (Fig. 5). These data showed clearly that DMEM promotes translocator secretion and suppresses effector secretion, while M9 has the opposite effect. In addition, our results indicated that induction in M9 mimics the effects of the loss of SepD and SepL function on TTS, suggesting that SepL and SepD regulate TTS hierarchy of translocators and effectors in response to environmental cues and growth conditions.

Calcium concentrations differentially modulate secretion, but not transcription, of translocators and effectors in CR, EPEC, and EHEC. We used several approaches to investigate what chemical components in DMEM and M9 media accounted for the differential effects on secretion of translocators and effectors. The two most obvious differences in chemical compositions between M9 and DMEM are $CaCl_2$ and NH_4Cl concentrations. While DMEM contains ~1.8 mM of Ca^{2+} and no NH_4^+, M9 has ~18 mM NH_4^+ and no Ca^{2+}. Addition of NH_4^+ into DMEM had a small effect on TTS, but did not change the overall secretion profile of EHEC (data not shown). To analyze the effect of calcium concentration on TTS, various concentrations (1 to 4 mM) of EGTA and the more calcium-specific chelator BAPTA were added to DMEM. As shown in Fig. 6, EHEC, EPEC, and CR all showed increased secretion of Tir and NleA, and much reduced secretion of translocators EspA, EspD, and EspB when EGTA and BAPTA were added to DMEM. The effect of the chelators was most evident when their concentration reached between 1.5 and 2 mM (Fig. 6). These results were as predicted by our calculation of free calcium concentration in bacterial growth media as a function of chelator concentration with the binding equation $0 = [Ca^{2+}]_{free} + ([chelator] - [Ca^{2+}]_{total} + K_d) \times \{[Ca^{2+}]_{free} - ([Ca^{2+}]_{total} + K_d)\}$ (see Materials and Methods). Based on this quadratic equation, $[Ca^{2+}]_{free}$ was unaffected until the concentration of the chelator approached 1 mM. A sharp inflection point was observed where the concentration of the chelator reached ~1.8 mM. At this point, $[Ca^{2+}]_{free}$ dropped from ~1 mM to ~1 μM, and the affinities of EGTA and BAPTA started to show differences (data not shown). The main prediction was that a calcium-dependent switch, if present, should be most evident when the concentration of the chelator was applied between 1.5 and 2 mM to DMEM. This prediction was supported by our results (Fig. 6). However, it should be noted that, unlike mutations in *sepL* and *sepD* (Fig. 1), the addition of chelators did not abolish the secretion of translocators. Furthermore, TTS of effectors by the *sepL* and *sepD* mutants was blind to calcium concentrations (Fig. 5 and data not shown).

Since it was possible that the chelators were not specific for calcium and may have affected the concentrations of other metal ions in DMEM, we further examined the role of calcium on the TTS profiles of the A/E pathogens with calcium-free DMEM. As shown in Fig. 6, EHEC, EPEC, and CR all displayed similar secretion profiles in calcium-free DMEM and DMEM containing 1.5 to 2 mM of calcium chelators. Adding 1 to 2 mM of $CaCl_2$ into calcium-free DMEM restored translocator secretion to the levels seen in regular DMEM and suppressed secretion of Tir and NleA. This switch from effector secretion to translocator secretion could also be seen when 1 to 2 mM of $CaCl_2$ was added into the modified, calcium-free M9 medium (data not shown). Taken together, these results indicated that calcium concentrations in culture media influence the levels of secreted translocators and effectors differentially and may act as a substrate switch signal for TTS of translocators and effectors.

The differential effect of calcium concentrations on TTS of translocators and effectors could be exerted during either gene expression or protein secretion. To distinguish between these

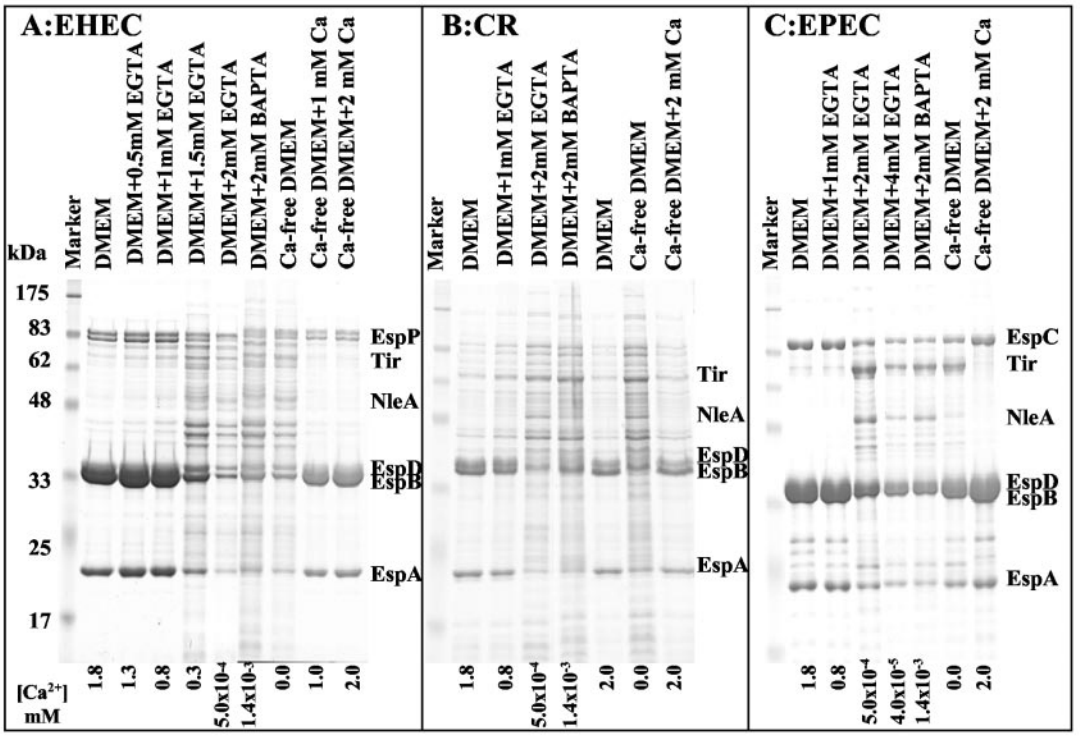

FIG. 6. Calcium concentrations in culture media affect TTS of translocators and effectors differentially in EHEC (A), CR (B), and EPEC (C). WT strains of EHEC, EPEC, and CR were grown in LB and then subcultured into regular DMEM, DMEM supplemented with various concentrations of EGTA and BAPTA, calcium-free DMEM, or calcium-free DMEM supplemented with 1 to 2 mM $CaCl_2$. Secreted proteins were concentrated from the bacterial culture supernatant and analyzed by SDS-PAGE and Coomassie blue G250 staining. Proteins secreted by an equal number of bacteria, as estimated by OD_{600} values, were loaded in each lane of the same gel. The locations of translocators (EspA, EspB, and EspD) and effectors (Tir and NleA), as well as EspC (EPEC) and EspP (EHEC), are indicated on the right. Predicted Ca^{2+} concentrations (in millimoles) in growth media are indicated at the bottom of each lane.

two possibilities, we analyzed by RT-PCR the mRNA levels of *tir* and *espB* isolated from CR, EPEC, and EHEC grown in regular DMEM, calcium-free DMEM, and calcium-free DMEM supplemented with 2 mM $CaCl_2$. No significant difference in transcription of *tir* and *espB* was detected (Fig. 2B), suggesting that calcium concentrations in growth media affect specifically secretion but not transcription of translocators and effectors.

SepL and SepD do not need each other for stability, and calcium concentrations do not affect SepL and SepD interactions. Because SepL and SepD interacted with each other (Fig. 4), it was possible that one of them served as a stabilizing chaperone for the other. Indeed, SepD exhibits certain characteristics of type III chaperones (9, 10; data not shown). To determine whether SepL and SepD need each other for stability inside bacteria, the 2HA-tagged CR *sepL* and *sepD* were introduced and expressed in various mutants of CR. As mentioned before, these constructs complemented their respective mutants, indicating that the C-terminal 2HA tag did not affect the normal function of SepL and SepD. It was observed that both SepL-2HA and SepD-2HA were present at similar levels in all the mutants tested (data not shown), including Δ*escN*, Δ*sepL*, Δ*sepD*, and the Δ*sepL*Δ*sepD* double mutant, suggesting that SepL and SepD did not need each other for stability. It also suggests that turnover of SepD or SepL may not be required for substrate switch during translocator and effector secretion.

We demonstrated that TTS by *sepL* or *sepD* mutants did not respond to calcium concentrations in growth media (Fig. 5 and data not shown). We also showed that the effect of chelating calcium in growth media partially mimicked that of mutations in *sepL* or *sepD* (Fig. 6). These results suggest that there may be a link between SepL/SepD and sensing of calcium concentrations. We therefore examined whether calcium concentrations affect SepL and SepD stability and their interactions. Similar levels of functional SepL-2HA and SepD-2HA were detected in WT CR as well as its Δ*escN*, Δ*sepL*, Δ*sepD*, and Δ*sepL*Δ*sepD* mutants in DMEM both in the presence and in the absence of $CaCl_2$ (data not shown). In addition, SepL-2HA and SepD-2HA were not type III secreted in either regular DMEM or calcium-free DMEM (data not shown). However, small amounts of SepL-2HA and SepD-2HA could be detected by Western blotting in culture supernatants of both WT CR and its Δ*escN* (type III) mutant grown in either regular DMEM or calcium-free DMEM (data not shown). This was consistent with our data that both SepL-2HA and SepD-2HA could be detected in bacterial outer membrane fractions (Fig. 3). We also examined whether calcium concentrations affected the interaction and complex formation between SepL and SepD. SepL and SepD bound to each other in vitro and formed a

stable protein complex (Fig. 4; data not shown). The presence or absence of various concentrations of $CaCl_2$ did not have any effect on the SepL-SepD interaction or their complex formation (data not shown).

DISCUSSION

Bacterial pathogens employing a TTSS often secrete a large number of proteins (4, 7, 9, 21). Due to the large number of proteins trafficking through the TTS apparatus, a secretion hierarchy is presumably needed for their orderly exodus. The secreted proteins consist of both effectors and translocators, with the latter needed for translocating effectors into host cells. It is therefore pivotal that pathogens secrete translocators before effectors. In addition, different effectors secreted may function at different stages of infection, and some of them may even have opposing functions, suggesting a need for hierarchical and temporal controls over their secretion. Although some progress has been made, TTS hierarchy remains poorly understood (1, 9, 45).

In this study, we analyzed the function of *sepL* and *sepD* genes in three prototypical strains of A/E pathogens, EPEC strain E2348/69, EHEC strain 86-24, and CR. We showed that mutations in *sepL* and *sepD* resulted in abolished secretion of translocators but significantly enhanced secretion of two effectors Tir and NleA. Furthermore, the *sepL* and *sepD* mutants exhibit highly similar secretion phenotypes in all three A/E pathogens. This strong conservation of the SepL and SepD function is consistent with the fact that SepL and SepD are among the most conserved proteins encoded by the LEE from different A/E pathogens, with SepL and SepD showing more than 90 and 88% identity, respectively (13, 19, 40, 44, 50).

The function of SepL and SepD is a matter of controversy in the literature. It has been reported that a *sepL* mutant of EHEC strain EDL933 secretes no translocators but increased p54/NleA (27), similar to our CR, EPEC, and EHEC *sepL* mutants (15; this study). While the manuscript was in preparation, O'Connell et al. reported that an EPEC *sepL* mutant secretes significantly reduced amount of translocators, but increased secretion of effectors by the mutant was not reproducibly seen (38). These phenotypes are different from those of our EHEC, EPEC, and CR *sepL* mutants (15, 27; this study). These discrepancies could be due to the different growth conditions used for TTS assays. O'Connell et al. grew EPEC strains in DMEM in an air incubator with shaking (33, 38). In our assays, however, EPEC strains were grown in DMEM in a CO_2 (5%) tissue culture incubator without shaking, conditions that promote TTS in EPEC and EHEC and have been used by many laboratories (24, 27, 31). Since our CR, EPEC, and EHEC *sepL* mutants all consistently secreted larger amounts of effectors than their respective WT strains, we believe that SepL plays the same role in all A/E pathogens and that mutations in *sepL* result in suppressed secretion of translocators and hypersecretion of effectors.

O'Connell et al. also reported functional analyses of an EPEC *sepD* mutant and concluded that SepD is an essential element of the TTS apparatus, since the *sepD* mutant did not secrete either translocators or effectors (38). These secretion phenotypes of their EPEC *sepD* mutant are drastically different from those of the CR, EHEC, and EPEC *sepD* mutants characterized in this study. Similar to the *sepL* mutants, all of the *sepD* in-frame deletion mutants we generated, including the EPEC *sepD* mutant, secreted no translocators, but the mutants did secrete significantly increased amounts of several effectors, including EspF, Tir, and NleA, as determined by different techniques (Fig. 1D) (15; data not shown). The discrepancies between our results and those of O'Connell et al. are difficult to reconcile but could be due to differences in experimental methodology. However, our results showed clearly that although required for translocator secretion, SepD is not essential for TTS in any of the three A/E pathogens tested, since its mutants are able to secrete all known effectors, including Tir, NleA, and EspF.

We have shown that SepL and SepD regulate secretion hierarchy of translocators and effectors in A/E pathogens. Many other TTSSs regulate the secretion of translocators and effectors differentially, although the mechanisms used can be variable. There are no strong homologues of SepL and SepD encoded by other TTSSs, except for SsaL of *Salmonella* pathogenicity island 2 (SPI-2). SsaL has more than 40% sequence similarity to SepL, and there is evidence that SsaL plays a role in regulating secretion of *Salmonella* SPI-2-encoded translocators (8). Two additional SPI-2-encoded proteins, SsaM and SpiC, appear to regulate the secretion of translocators and effectors similar to SepL and SepD (49). InvE of the *Salmonella* SPI-1-encoded TTSS may also play a role similar to that of SepL or SepD. The *invE* mutation significantly reduces, but does not abolish, TTS of translocators; it slightly increases TTS of some effectors (28). These phenotypes are different from the *sepL* and *sepD* mutants that have abolished secretion of translocators and significantly enhanced secretion of effectors. It has been shown that InvE interacts with a protein complex formed by translocators and their cognate chaperone, but it does not interact efficiently with the individual components of this complex (28). We are investigating the potential interactions of SepL and SepD with the translocators EspA, EspB, and EspD and their chaperones.

In *S. flexneri*, certain translocators such as IpaB can outcompete effectors for the secretion channel, as mutants of these translocator genes exhibit deregulated and increased secretion of a number of proteins, many of which are effectors (4, 39). Our results show that SepL and SepD are required for the secretion of translocators by A/E pathogens. The significantly enhanced secretion of effectors seen in the *sepL* and *sepD* mutants could therefore be the result of reduced competition for the secretion channel, due to the blocked translocator secretion. However, this is not the case, since a triple deletion mutant (Δ*espADB)* in CR of all the translocator genes secretes normal, not increased, levels of effectors (Fig. 1A); this suggests that SepL and SepD also play a role in suppressing effector secretion under conditions favorable for translocator secretion.

In addition to the bacterial genetic elements discussed above, environmental factors also influence the secretion hierarchy of translocators and effectors. This has been intensively studied in *Yersinia* spp., especially the role of calcium (10). TTS of *Yersinia* Yops occurs in a sequential manner, secreting first the translocators YopBD and followed by the effectors YopEHJMOT (29). Secretion of the translocators is activated by albumin and other serum proteins in the presence of calcium, whereas secretion of the effectors is triggered by a low-calcium environment (10, 29). How calcium chelation triggers secretion of effector Yops is still not understood. It has been proposed that *Yersinia* species may use the TTS apparatus, which forms a hollow conduit connecting the bacteria to the host cell cytoplasm, as a tool to sense

and measure host intracellular calcium concentration to signal the transport of Yops (29, 41). According to this hypothesis, the relatively high calcium concentration (about 2.5 mM) in the mammalian host extracellular fluid (10) suppresses TTS of the Yops. On the other hand, the calcium concentration in both the eukaryotic host cell cytoplasm and the bacterial cytosol is estimated to be low, ranging from 100 and 300 nM, and most of the calcium in eukaryotic cells is sequestered in the endoplasmic reticulum (3, 16). Upon contact with host cells, the needle complexes of the *Yersinia* TTSS, which are assembled prior to host cell contact, are inserted into eukaryotic cells and allow the bacteria to sense the change in calcium concentrations, thereby activating Yop secretion.

In this report, we have confirmed the observation that calcium chelation reduces TTS of the translocators and increases the secretion of effector Tir in EPEC strains (24, 25) and analyzed the effect of calcium on TTS in EPEC in greater detail. In addition, we extended our observations to other important A/E pathogens, namely EHEC and CR. Our results suggest that regulation of secretion by calcium is a conserved mechanism for TTSSs. In calcium-rich media, A/E pathogens secrete mostly translocators and very small amounts of effectors. When calcium is limited, translocator secretion is significantly reduced, while effector secretion is greatly enhanced (Fig. 5 and 6). While calcium concentrations in culture media have opposite effects on the secretion of translocators and effectors in WT A/E pathogens, their *sepL* and *sepD* mutants are calcium blind and secrete no translocators but significantly increased effector levels both in the presence and in the absence of calcium (Fig. 1 and 5). This suggests that SepL and SepD may be linked to calcium sensing. It is worth noting that in *Yersinia*, secretion of translocators occurs regardless of calcium concentrations and that calcium depletion triggers secretion of all Yops, both translocators and effectors. In addition, calcium-blind mutants in *Yersinia*, such as a *yopN* mutant, secrete translocators as well as effectors (10, 29, 41), unlike the *sepL* and *sepD* mutants of A/E pathogens that secrete greatly increased effectors but no translocators. Our results suggest that A/E pathogens use calcium concentrations not only to regulate TTS of effectors, but also to control the secretion hierarchy of translocators and effectors.

Based on these observations, we have modified the calcium-signaling model in *Yersinia* suggested by Schneewind and colleagues (29, 41) and propose that TTS in A/E pathogens occurs in two phases during infection. In the first phase, when the A/E pathogens enter their host via ingestion, host gastrointestinal conditions activate LEE gene expression, leading to the assembly of TTS apparatus in the bacterial membranes. In the presence of calcium in the extracellular fluid of the intestinal lumen, the bacteria first secrete the translocators EspA, EspB, and EspD, allowing the assembly of a translocation pore (translocon) on the host cell membrane. The translocon and the TTS apparatus form a conduit connecting the bacterial cytosol and the host cytoplasm, where calcium is limited. In the second phase of TTS, the bacteria detect the low-calcium environment in the host cell via the translocon and the TTS apparatus, which signals the docking of the bacteria onto a host cell. Low calcium suppresses secretion of the translocators, and activates secretion of the effectors. This differential effect of calcium on translocator secretion and effector secretion ensures that translocators are secreted before effectors and that effectors are efficiently translocated into host cells. The regulation of secretion of translocators and effectors is likely to occur at the posttranscriptional level, since we cannot detect significant differences in expression of effectors and translocators by RT-PCR in WT A/E pathogens and their *sepD*, *sepL*, and *escN* mutants or when the bacteria are grown in calcium-rich or calcium-free media (Fig. 2). Our results are consistent with published reports showing that secretion of translocators in EHEC and EPEC is regulated by growth media at the posttranscriptional level (12, 24, 42).

It is not yet clear what senses calcium in A/E pathogens. SepL and SepD are good candidates for this role, since loss of either protein renders the TTS of effectors insensitive to calcium concentration (Fig. 5). As single mutations in *sepD* or *sepL* have the same effect on TTS as their double mutant (15), SepD and SepL may perform their function as a complex. Indeed, we and others have shown that SepL and SepD interact with each other by several independent methods (Fig. 4) (38). It does not appear that either SepL or SepD acts as a chaperone, since the stability of either protein is not affected by the absence of the other (data not shown). Both SepL and SepD have been shown to associate with the bacterial membranes (Fig. 3) (27). We propose that SepL and SepD together serve as gatekeepers, instead of as plugs, of the TTSS, since mutations in either *sepL* or *sepD* still selectively allow the secretion of effectors to occur. However, we have so far failed to establish a direct link between SepL/SepD and calcium sensing. Calcium does not affect the interaction between SepL and SepD or their secretion and stability in bacteria (data not shown). It is still possible that calcium concentrations affect interaction or engagement of SepL and SepD with an as-yet-unidentified component(s) of the TTS apparatus. It should be pointed out that low calcium may not be the only signal for secretion substrate switch from translocators to effectors, as calcium-free media significantly reduce, but do not abolish, TTS of translocators, unlike mutations in *sepL* or *sepD*. These data suggest that, in addition to calcium, other environmental signals also play a role in regulating TTS. We are currently exploring these possibilities. We believe that understanding the molecular mechanism of how SepL and SepD control the secretion of translocators and regulate the secretion hierarchy of translocators and effectors should have broad implications for other TTSSs.

ACKNOWLEDGMENTS

We thank R. A. Edwards for providing strains and plasmids, F. Roche for sharing unpublished data on in silico analysis of EHEC SepD, and C. Lupp for reviewing the manuscript.

B.B.F. is supported by grants from the Canadian Institutes of Health Research (CIHR), the Howard Hughes Medical Institute (HHMI), and the Canadian Bacterial Diseases Network (CBDN). J.L.P. is funded by Dirección General de Asuntos del Personal Académico (DGAPA), Consejo Nacional de Ciencia y Tecnología (CONACyT), and HHMI. N.C.J.S. is funded by CIHR, the Burroughs Wellcome Foundation, and HHMI. B.B.F., J.L.P., and N.C.J.S. are HHMI International Research Scholars. B.B.F. is the UBC Peter Wall Distinguished Professor and a CIHR Distinguished Scientist.

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Editor: A. D. O'Brien